	L #	Hits	Search Text	DBs	Time Stamp
1	L1	510	galactosyltransferase$1 or galactosyl adj transferase$1	USPAT; US-PGPUB	2002/12/05 12:50
2	L2	6007	gb3 or cd77 or globotriaosylceramide	USPAT; US-PGPUB	2002/12/05 12:51
3	L3	1	2 adj synthase$1	USPAT; US-PGPUB	2002/12/05 12:51
4	L4	18	1 and 2	USPAT; US-PGPUB	2002/12/05 12:53

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TITLE: Compositions and methods for treatment of neoplastic disease

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INVENTOR-INFORMATION:
NAME CITY STATE COUNTRY RULE-47
Terman, David S. Pebble Beach CA US

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ABSTRACT:

The present invention comprises compositions and methods for treating a tumor or neoplastic disease in a host, The methods employ conjugates comprising superantigen polypeptides, nucleic acids with other structures that preferentially bind to tumor cells and are capable of inducing apoptosis. Also provided are superantigen-glycolipid conjugates and vesicles that are loaded onto antigen presenting cells to activate both T cells and NKT cells.
Cell-based vaccines comprise tumor cells engineered to express a superantigen along with glycolipids products which, when expressed, render the cells capable of eliciting an effective anti-tumor immune response in a mammal into which these cells are introduced. Included among these compositions are tumor cells, hybrid cells of tumor cells and accessory cells, preferably dendritic cells.
Also provided are tumoricidal T cells and NKT cells devoid of inhitory receptors or inhibitory signaling motifs which are hyperresponsive to the the above compositions and lipid-based tumor associated antigens that can be administered for adoptive immunotherapy of cancer and infectious diseases.

---------- KWIC ---------

Detail Description Paragraph - DETX:

[0112] The synthetic pathway for neutral glycosphingolipids in eukaryotic cells is known. Glucosylceramide (GlcCer) is the precursor of lactosylceramide

(LacCer), which leads, in order, to Gb3 and globotetraosylceramide (Gb4). Different Golgi enzymes are responsible for addition of monosaccharides from nucleotide-sugar donors in each step of the pathway. **Globotriaosylceramide synthase** (UDP-galactose:lactosylcerami- de .alpha.1-4-galactosyltransferase) has been purified. In the cytoplasm, the a-subunit of the Shiga toxin or VT is processed by a trypsin-like cleavage. The "activated" 27-kDa a-subunit inactivates 60S ribosomes by depurination of a single nucleotide in 28S rRNA, rendering ribosomes incapable of carrying out peptide elongation.

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Also provided are tumoricidal T cells and NKT cells devoid of inhitory receptors or inhibitory signaling motifs which are hyperresponsive to the the above compositions and lipid-based tumor associated antigens that can be administered for adoptive immunotherapy of cancer and infectious diseases.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0037] For in vivo immunization, tumor cells are transfected with nucleic acids encoding SAgs together with a carbohydrate modifying enzyme such as

.alpha.-**galactosyl transferase** to produce the .alpha.-Gal epitope, Staphylococcal hyaluronidase, Streptococcal capsular polysaccharide, Staphylococcal erythrogenic toxin, Staphylococcal Protein A, Staphylococcal .beta.hemolysin, Staphylococcal coagulase, costimulants such as B7-1 and B7-2, chemoattractants and chemokines. SAgs are also cotransfected into tumor cells with gene clusters encoding the biosynthesis of highly immunogenic microbial Lipid A, membrane or capsular polysaccharides, lipoproteins and peptidoglycans. Nucleic acids are useful when transfected alone. However combinations are preferred. The cotransfection into tumor cells of the SAg-encoding nucleic acid together with the nucleic acids encoding .alpha.Gal or GalCer biosynthesis is particularly useful. The cotransfection into tumor cells of the nucleic acid encoding SAg with nucleic acids encoding Staphylococcal erythrogenic toxins and hyaluronidase allows the transfected tumor cells to simulate the in vivo inflammatory activity of a Staphylococcus or leukocyte or macrophage by secreting enzymes and toxins which induce a sterile cellulitis in tumor sites.

Brief Description of Drawings - Table CWU - DRTL:

1TABLE I Therapeutic Constructs And Preferred Conditions Of Use I. CELLS: Tumor Cells, DCs or DC/Tumor Cell Hybrids (DC/tc) USE: In vivo and Ex vivo PURPOSE A. In Vivo Preventative or Therapeutic Vaccine (Established Tumor) Accomplish by transfecting or co-transfecting with nucleic acid encoding superantigen plus one or more of the following: 1. Superantigens 2. Enzyme that modifies carbohydrate to induce Gal or GalCer epitope expression 3. Functional hyaluronidase from microbial or human sources 4. Staphylococcal or streptococcal erythrogenic toxin 5. Staphylococcal protein a or a domain thereof 6. Staphylococcal hemolysin and functional microbial toxins 7. Functional microbial or human coagulase 8. Costimulatory protein 9. Chemoattractants 10. Chemokines 11. Nucleic acids encoding biosynthesis of lipopolysaccharides 12. Nucleic acids encoding biosynthesis of glycosylceramides 13. Nucleic acids encoding biosynthesis of microbial membrane or capsular lipoproteins and polysaccharides 14. Oncogenes, amplified oncogenes and transcription factors 15. Angiogenic factors and receptors 16. Tumor growth factor receptors 17. Tumor suppressor receptors 18. Cell cycle proteins 19. Heat-shock proteins, ATPases and G proteins 20. Proteins engaged in antigen processing, sorting and intracellular trafficking 21. Inducible nitric oxide synthase (iNOS) 22. apolipoproteins (e,g,. Lp(a)) transfected into tumor cells & sickled erythrocytes used for targeting tumor microvasculature 23. LDL and oxyLDL receptors (e.g., SCEP receptor) transfected into tumor cells and sickled erythrocytes & used for targeting to tumor microvasculature B. Ex Vivo Immunization of T and/or NKT cells to Produce Tumor Specific Effector Cells (for Adoptive Immunotherapy)* Accomplish by (i) transfecting or co-transfecting tumor or accessory cells with nucleic acid encoding the following, or (ii) providing immobilized molecules or receptors that present the following: 1. Superantigen 2. Superantigen receptor and transcription factor with bound superantigen 3. CD1 receptor binding and/or expressing superantigen-glycosyl ceramide complex 4. CD14 receptor binding or expressing superantigen-lipopolysaccharide or superantigen- peptidoglycan complex 5. Mannose receptor binding glycosylated superantigen 6. Glycophorin receptor 7. Superantigen-tumor peptide(s) complex on MHC or CD1-bearing APC in soluble or immobilized form C. Therapeutic Molecules or Complex Applied to Transfected or Untransfected Tumor

cells or Accessory Cells; or MHC class I, class II, CD1, Superantigen receptor or CD14 receptor: 1. Superantigen (wherein cell may express Gal) 2. Glycosylated superantigen 3. Superantigen complex with a. glycosyl ceramide b. lipopolysaccharide c. peptidoglycan d. mannan proteoglycan e. muramic acid f. tumor peptide g. glycosylceramides with terminal Gal(.alpha.1-4)Gal e.g. globotriosylceramide and galabiosylceramide h. Conjugates of SAg-(Gb2 or **Gb3** or Gb4) i. Conjugates of SAg-(Gb2 or **Gb3** or Gb4)-CD1 j. GPI anchored conjugates: SAg-GPI-(Gb2 or **Gb3** or Gb4) l. GPI anchored conjugates: SAg-GPI-(Gb2 or **Gb3** or Gb4)-CD1 m. Conjugates of SAg polypeptide or nucleic acid with Verotoxin n. Conjugates of SAg polypeptide or nucleic acid with Verotoxin A or B subunit o. Conjugates of SAg polypeptide or nucleic acid with IFN.alpha. receptor peptides homologous to verotoxin p. Conjugates of SAg polypeptide or nucleic acid with CD19 peptides homologous to verotoxin q. Conjugates of SAg polypeptide or nucleic acid with Arg-Gly-Asp or Asn-Gly-Arg r. Conjugates of SAg polypeptide or nucleic acid with LDL, VLDL, HDL s. Conjugates of SAg polypeptide or nucleic acid with Apolipoproteins (e.g., Lp(a), apoB- 100, apoB-48, apoE) 9. Conjugates of SAg polypeptide or nucleic acid with oxyLDL, oxyLDL mimics, (e.g., 7.beta.-hydroperoxycholesterol- , 7.beta.-hydroxycholesterol, 7-ketocholesterol, 5.alpha.- 6 .alpha.-epoxycholesterol, 7.beta.-hydroperoxy-choles-5-en-3.beta.-ol, 4-hydroxynonenal (4-HNE), 9-HODE, 13-HODE and cholesterol-9-HODE) 10. Conjugates of SAg polypeptide or nucleic acid with oxyLDL byproducts (e.g. lysolecithin, lysophosphatidylcholine, malondialdehyde, 4- hydroxynonenal) v. LDL & oxyLDL receptors (e.g., LDL oxyLDL, acetyl-LDL, VLDL, LRP, CD36, SREC, LOX-1, macrophage scavenger receptors) as polypeptide or nucleic acid alone or with SAg polypeptide or nucleic acid intratumorally 11. phytosphingosine, -GPI-phytosphinosine, 12. tumor associated lipid antigens, glycolipid, proteolipid, glycosphingolipid, sphingolipid with inositolphosphate-containing head groups, phytoglycolipids, mycoglycolipids. GPI-sphingosines or lipids y. sphingolipids with inositolphosphate-containing head groups having the general structure: ceramide-P-myoinositol-X with X referring to polar substituents comprising ceramide-p-inositol-mannose, inositol-1-P-(6)mannose(.alpha.1,2inositol-1P-(l) ceramide, (inositol-P)2-ceramide, inositol-P-inositol-P-ceramide, inositol-P-inositol-P-ceramide. z. tumor associated glycan antigens consisting of peptidoglycans or glycan phosphotidyinositol (GPI) structures. z chemokine receptors polypeptide or nucleic acid II. CELLS: Specialized Tumor Specific Effector Cells (T and/or NKT Cells) USE: Adoptive Immunotherapy In Vivo PURPOSE: A. CD44 Expression on T cells or NKT Accomplished by: (i) Superantigen stimulation; and/or (ii) transfection with nucleic acid encoding CD44 and/or (iii) transfection with nucleic acid encoding glycosyltransferase B. Chimeric TCR with: Invariant a chain site for binding GalCer and V.beta. chain site for binding superantigen C. Dual TCR V.beta. chains with sites for superantigen binding D. T cells or NKT cells with overexpressed V.beta. region specific for a given superantigen E. T cells or NKT cells with lowered signal transduction threshold III. MOLECULES: Superantigen mimics#USE: In Vivo Administration A. Superantigen receptor-binding oligonucleotides B. Superantigen oligonucleotide-peptide conjugate Oligo nucleofide is specific for superantigen receptor on tumor cells Peptide has deleted class II binding site and intact TCR binding site C. Phage displayed integrin ligand on tumor neovasculature - carrier for superantigen- encoding nucleic acid. IV. CARRIERS: for nucleic acid encoding superantigen USE Transfection of Tumors In vivo A. Sickled erythrocytes that

target tumor neovasculature B. Phage displayed tumor neovascular integrin and superantigen receptor carrying superantigen nucleic acids V. CARRIERS: constructed to co-express superantigen conjugates or complexes with: Glycosylceramide .alpha.Gal Lipopolysaccharides Peptidoglycans USE Transfection of Tumor Cells and/or DCs and/or DC/tc's - in vivo or ex vivo. A. Liposomes B. Proteosomes

Detail Description Paragraph - DETX:

[0053] Cells transfected with nucleic acid encoding a SAg may also express a tumor associated antigen that is potentially present on host cancer cells. For example, nucleic acid encoding a known tumor antigen are transfected into the SAg-containing cell, or a tumor cell that endogenously contains many different tumor antigens are transfected with SAg-encoding nucleic acid. In the latter case, additional nucleic acids encoding other polypeptides are transfected into the tumor cell. For example, nucleic acid encoding a carbohydrate modifying enzyme such as .alpha.1,3-**galactosyltransferase,** adhesion molecule, costimulatory molecule such as B7-1 and B7-2, MHC class I molecule and/or MHC class II molecule are cotransfected into tumor cells together with nucleic acid encoding a SAg.

Detail Description Paragraph - DETX:

[0069] Cells are transfected in vivo or in vitro. When transfected in vivo, the cells are of host origin. When transfected in vitro, the cells are autologous, allogeneic, or xenogeneic to the host to provide additional immunogenicity. In addition to being transfected with nucleic acid encoding a SAg, the cells are transfected with nucleic acid encoding any other polypeptide including, without limitation, a **galactosyltransferase,** staphylococcal hyaluronidase and/or erythrogenic toxin, streptococcal capsular polysaccharide, CD44, tumor antigen, costimulatory molecule such as B7-1 and B7-2, adhesion molecules, MHC class I molecule and/or MHC class II molecule. Nucleic acids encoding the molecules are cotransfected with the SAgs. But for others, including but not limited to Staphylococcal hyaluronidase, erythrogenic toxin, Streptococcal capsular polysaccharide and CD44 genes, the nucleic acids encoding the SAgs are fused to other nucleic acids resulting in expression of a fusion protein.

Detail Description Paragraph - DETX:

[0111] SAgs have an affinity for glycosphingolipids especially those with terminal or subterminal Gal(.alpha.1-4)Gal residues. Such residues are expressed on tumor cells as Gal(.alpha.1-4)Gal(62 1-4)GlcCeramide (**globotriaosylceramide or Gb3**) and Gal(.alpha.1-4)GalCeramide (galabiosylceramide or Gb2). **Gb3** and Gb2 also known as **CD77,** Burkitt's lymphoma antigen, and the human blood group p.sup.k antigen are the natural receptors for Shiga toxins and VT's. Shiga toxin, a 69-kDa complex of proteins comprised of five b-subunits (7 kDa each) and one a-subunit (30 kDa) has high affinity for the terminal digalactose of **Gb3** or Gb2. Methods for their preparation and isolation are described in Example 41. Once bound to the tumor

cell, these toxins are internalized and induce apoptosis.

Detail Description Paragraph - DETX:

[0112] The synthetic pathway for neutral glycosphingolipids in eukaryotic cells is known. Glucosylceramide (GlcCer) is the precursor of lactosylceramide (LacCer), which leads, in order, to **Gb3** and globotetraosylceramide (Gb4). Different Golgi enzymes are responsible for addition of monosaccharides from nucleotide-sugar donors in each step of the pathway. **Globotriaosylceramide** synthase (UDP-galactose:lactosylcerami- de .alpha.1-4-**galactosyltransferase**) has been purified. In the cytoplasm, the a-subunit of the Shiga toxin or VT is processed by a trypsin-like cleavage. The "activated" 27-kDa a-subunit inactivates 60S ribosomes by depurination of a single nucleotide in 28S rRNA, rendering ribosomes incapable of carrying out peptide elongation.

Detail Description Paragraph - DETX:

[0113] The present invention provides therapeutically active soluble complexes comprising SAg and glycosphingolipids which have terminal or subterminal Gal(.alpha.1-4)Gal residues and Shiga toxin receptors **Gb3** and Gb2, (collectively referred to as "GTSG1-4"). These complexes include but are not limited to SAg-GPI-GTSG1-4 complexes, and synthetic and functional derivatives thereof Such structures appear naturally on surfaces of certain tumor cells such as astrocytoma, Burkitt's lymphoma and ovarian carcinoma. Methods of preparing and isolating glycosylceramides and VTs are given in Examples 41 and 55.

Detail Description Paragraph - DETX:

[0116] Additional immunogenic complexes comprising SAgs bound to tumor cells, DCs DC/tc constructs expressing surface Gb2 and **Gb3** or other glycosphingolipids containing terminal Gal(.alpha.1-4)Gal are prepared by transfecting these cells with nucleic acids encoding a SAg. The transfected cell expresses the SAg in the context of the glycosphingolipid comprising the terminal or subterminal Gal(.alpha.1-4)Gal moiety. Alternatively, free or GPI linked glycolipids containing SAg peptides or polypeptides bind to tumor cells or accessory cells in tissue culture (Section 38). The expression of **Gb3** and Gb2 on tumor cells is optionally upregulated by various cytokines, including IFN.alpha. and TNF .alpha., before contacting the SAg

Detail Description Paragraph - DETX:

[0120] The present invention contemplates the induction of apoptosis in tumor cells expressing Gb2 and **Gb3** (or other glycosphingolipids containing terminal Gal(.alpha.1-4)Gal) by using free SAgs, conjugates and fused DNA that comprises SAg, SAg peptide or SAg-encoding DNA fused to intact VT or to VT A or B chains. Preparation of these conjugates and fusion proteins from their corresponding DNA, polypeptides or functional derivatives is provided in Examples 1 and 5. These conjugates induce apoptosis by binding to tumor cell glycosphingolipid

receptors having terminal Gal(.alpha.1-4)Gal. Methods of assessing tumor cell apoptosis are in Example 44. CD19 or IFNa peptide sequences and generic carbohydrate recognition domains which bind Gal(.alpha.1-4)Gal structures are also useful. CD19, a B-cell restricted differentiation antigen, naturally binds to **Gb3** and Gb2 on the cell surface which incudes apoptosis. CD19 has VT-like sequences in the N-terminal extracellular domain (NBRF protein data bank) that have 41%, 34% and 37% sequence identity to VT1, VT2, and VT2e B subunits, respectively. When compared to a consensus VT B sequence, the CD19 sequences show 49% identity. Binding of these peptide sequences to membrane Gal(.alpha.1-4)Gal-containing glycolipids facilitates receptor mediated induction of apoptosis.

Detail Description Paragraph - DETX:

[0121] The IFNa .alpha. receptor has a 63-kDa extracellular peptide with regions of amino acid identity to domains in the VT B subunit implicated as Gb2/Gb3 binding sites. The preferred targets of the above conjugates on tumor cells are the naturally expressed Shiga toxin receptors **Gb3** and Gb2 with a terminal Gal(.alpha.1-4)Gal. Astrocytomas and Burkitt's lymphomas are the preferred tumors as they naturally express glycosphingolipid receptors. However, any tumor expressing the appropriate receptor is appropriate. Tumor cells which express either engineered or natural functional derivatives, or mutants of these glycosphingolipid receptors, are also useful. Receptor expression on the target cells is optionally upregulated by cytokines such as IFN.gamma. and TNF.alpha.. Tumor cell sensitivity to the cytotoxic effects of a VT is enhanced by administration of interleukin-1.beta. before the addition of the conjugates. Tumor cells which do not naturally display **Gb3** or Gb2 acquire these structures by transfer from free, soluble structures or liposomes which express the missing glycosphingolipid receptor (Section 38, Example 5). The reconstituted tumor cells bearing the appropriate glycolipid receptors are thus targeted for apoptosis by the above constructs and conjugates.

Detail Description Paragraph - DETX:

[0129] A second method for creating cells that overexpress the foregoing glycolipids uses monensin or brefeldin which block additional glycosylation and sialylation of the -galactosylceramides, so that the mono- and digalactosylceramides accumulate in the cell. A third approach employs cells from patients with Fabry's disease. These cells are genetically deficient in the -galactosidase so they naturally accumulate -galactosylceramides. In a forth technique, an -galactosidase deficiency is induced in the target cell so that -galactosylceramides accumulate. In a fifth approach, the **-galactosyltransferase** is transfected Fabry's disease cells, thereby adding to the usual accumulation due to the catabolic enzyme deficiency. Such cells should have massive accumulations of -galactosylceramides. In a sixth approach, the desired mono- or diglycosylceramide expressed on liposome surfaces are transferred to tumor cells lacking these structures by co-culture and employment of fusion techniques given in example 5.

Detail Description Paragraph - DETX:

[0142] The a .alpha.Gal epitope is expressed by endothelial cells in xenografts such as pig organs is a major antigenic target causing hyperacute organ rejection in human transplant patients. This hyperacute rejection appears to involve a complement dependent mechanism that occurs within a few minutes. An .alpha.1-3-**galactosyltransferase,** is an enzyme capable of producing .alpha.1-3-galactose-.beta.1-4-N-acetylglucosamine moiety by adding a terminal galactose residue to a subterminal galactose residue via an .alpha.1-3 linkage. In addition, the .alpha.1-3-**galactosyltransferase** is not expressed by human and certain primate cells. Humans contain xenoreactive natural antibodies that recognize .alpha.Gal. For example, anti-Gal antibodies bind to pig endothelial cells that express the Gal epitope. These anti-Gal antibodies are naturally occurring IgM antibodies recently found to be present in large amounts in human serum. Surface expression of the .alpha.Gal epitope on tumor cells is achieved by transfecting a cell with a cDNA clone encoding the .alpha.1-3-**galactosyltransferase**. While tumor cells are the preferred cells for transfection, other cells such as accessory cells or immunocytes are also contemplated as being within the scope of this invention.

Detail Description Paragraph - DETX:

[0143] Nucleic acids encoding .alpha.1-3-**galactosyltransferase** polypeptides are known (Sandrin, M S et al., Proc. Natl. Acd. Sci. USA 90: 11391-11395 (1993)). A cDNA clone encoding murine .alpha.1-3-**galactosyltransferase** is prepared using the known sequence of this protein and the polymerase chain reaction (PCR) technique (Dabrowski, P L et al., Transplant. Proc. 26: 1335-1337 (1994). Briefly, two oligonucleotide primers are synthesized: (SEQ ID NO:30) 5'-GAATTCAAGCTTATGATCACTATGCTTCAAG-3', which is a sense primer that encodes the first 6 amino acids of the mature .alpha.1-3-**galactosyltransf-erase** and contains an HindIII restriction site; and (SEQ ID NO:31) 5'-GAATTCCTGCAGTCAGACATTATTCTAAC-3', which is an anti-sense primer that encodes the last 5 amino acids of the premature .alpha.1-3-galactosyitran- sferase and contains an in-frame termination codon and PstI restriction site. These primers amplify a 1185 bp fragment from a C57BL/6 spleen cell cDNA library that is subsequently purified, digested with HindIII and PstI (Pharmacia LKB) restriction endonucleases, and directionally cloned into HindIII/Pst I-digested expression vector such as CDM8 vector. After verifying the correct sequence, the .alpha.1-3-**galactosyltransferase**-cont- aining expression vector is transfected into heterologous cells such as COS cells to confirm activity. Activity can be confirmed by testing transfected cells for .alpha.Gal expression using the IB4 lectin (Sigma) of Griffonia simplicifolia that binds to Gal residues.

Detail Description Paragraph - DETX:

[0144] In the preferred mode, cells transfected with nucleic acids encoding a SAg are co-transfected with nucleic acids that encode an .alpha.-**galactosyltransferase**. Alternatively, nucleic acids encoding the transferase are transfected into a separate cell population which is coadministered with the SAg transfected cell population.

Detail Description Paragraph - DETX:

[0145] The SAg-encoding nucleic acid can be transfected into cells which already express .alpha.Gal epitope. In addition, any cell can be transfected with the **-galactosyltransferase**-encoding nucleic acid. For example, .alpha.Gal-negative human tumor cells or tumor cell lines such as melanoma or adenocarcinoma are transfected with nucleic acid encoding the .alpha.**-galactosyltransferase**. Tumor cells transfected with **-galactosyltransferase**-encoding nucleic acid express the .alpha.Gal on their surface and are rapidly rejected when administered to a host with preexisting .alpha.Gal specific antibodies. Methods of transfection are given in Example 1.

Detail Description Paragraph - DETX:

[0147] The ability of .alpha.Gal-transfected tumor cells to induce rejection is demonstrated by implantation into severely compromised immune deficient (SCID) mice that have been reconstituted with human T and B cells and transfused with normal human plasma containing the naturally occurring human antibodies specific for the .alpha.Gal epitope. In this case, tumor cells transfected with **-galactosyltransferase**-encodin- g nucleic acid is rejected while untransfected cells are not. Similarly, tumor cells transfected with .alpha.**-galactosyltransferase**-encoding nucleic acid is rejected when implanted into species such as humans which synthesize antibodies to the .alpha.Gal epitope compared to untransfected control tumor cells that are unaffected by the treatment.

Detail Description Paragraph - DETX:

[0148] For example, pretreatment with 10.sup.5-10.sup.7 .alpha.**-galactosyltransferase** transfected tumor cells subcutaneously followed by implantation of untransfected tumor cells prevents the outgrowth of untransfected malignant tumor cells. Hence, the **-galactosyltransferase** transfected tumor cells function as a vaccine. Further, **-galactosyltransferase** transfected cells implanted into animals after untransfected tumors are established induce rejection of an established untransfected tumor.

Detail Description Paragraph - DETX:

[0149] To test for the presence of .alpha.Gal on a cell surface, .alpha.1-3 **galactosyltransferase** knockout mice that do not express the .alpha.Gal antigen are used. The .alpha.1-3 **galactosyltransferase** knockout mice are described elsewhere (Tearle et al., Transplantation 61:13-19 (1996) and Shinkel et al., Transplantation 64:197-204 (1997)). A syngeneic tumor cell that is .alpha.Gal negative such as B16 melanoma variants is transfected with nucleic acids that encode a given carbohydrate modifying enzyme. These transfected cells are then implanted into the knockout mouse that received plasma containing .alpha.Gal

specific antibodies. Tumors do not grow in animals containing .alpha.Gal specific antibodies if the .alpha.Gal epitope is expressed.

Detail Description Paragraph - DETX:

[0151] .alpha.Gal negative transgenic animals are prepared which are useful for testing Gal expressing tumors. To produce these animals, nucleic acids encoding .alpha.Gal fucosyltransferase are transfected into .alpha.Gal positive mice. The fucosyltransferase dominates the usage of substrate N-acetyllactosamine and precludes **-galactosyltransferase** from utilizing this substrate. The transgenic mice do not express a-Gal on the cell surface. In this way, transgenic mice with the H antigen rather than the Gal antigen develop. Transgenic guinea pigs producing minimal .alpha.Gal are also created in this way. These animals are used as models for testing their capacity to reject syngeneic .alpha.Gal positive tumors. These systems also permit the testing of .alpha.Gal specific antibodies for anti-tumor effects after they are passively infused into animals bearing Gal positive tumors.

Detail Description Paragraph - DETX:

[0153] Fucosylated glycolipids such as B group antigens, Lewis blood group antigens, and L-selectin ligands are converted to the .alpha.Gal epitope using the appropriate sialidases and glycosyltransferase enzymes. For example, a desialylating enzyme is introduced into B group antigen expressing cells such that the .alpha.-1-3-linked galactose is exposed and now recognized by .alpha.Gal antibodies. Mild acid treatment to remove the branching fucose residues on the fucosylated B antigen is used to expose the .alpha.1,3galactose residues. Alternatively, cells expressing the B antigen or selectin antigen are transfected with **-galactosyltransferase**-encoding nucleic acid that competes successfully with fucosyltransferases for N-acetyl-lactosamine substrate and preferentially expresses the .alpha.Gal epitope.

Detail Description Paragraph - DETX:

[0155] Co-transfection of tumor cells with nucleic acid encoding SAg and nucleic acid encoding a **galactosyltransferase,** sialidase, and/or glycosyltransferase results in expression of SAg, GalCer, .alpha.Gal, or other glycolipids on the cell surface. These tumor cells are used to stimulate T or NKT cells ex vivo to produce a population of tumor specific effector cells which are deployed for adoptive immunotherapy of cancer.

Detail Description Paragraph - DETX:

[0213] The Shiga toxin of Shigella dysenteriae and Shiga-like toxins of E. coli (verotoxins) are a family of related toxins which have similar amino acid sequences and biological activities. The A subunit of Shiga toxin has a molecular mass of 31 kDa which associates with five to the 7 kDa B subunits. The A subunits is proteolytically cleaved into A1 and A2. It is the A1 fragment which is biologically active. The host cell receptor for Shiga toxin

is the glycolipid Gal(.alpha.1-4)Gal(.beta.1-4) GlcCeramide (globotriosylceramide; **Gb3**) and for Shiga-like toxin I (SLTI) and SLTII of E. coli is Gal(.alpha.1-3)GalCeramide (Galabiosylceramide). The binding specificity is dependent on both sugars residues and the lipid moiety. The Shiga toxin is known to inhibit protein synthesis. It is a RNA N-glycosidase enzyme whose site of action is the 60S ribosomal subunit. The toxins remove an adenine base from position 4324 on the aminoacyl -transfer RNA binding site of 28S ribosomal RNA hence preventing peptide length elongation. The effect on protein synthesis is similar to that of diphtheria toxin and Pseudomonas aeruginosa exotoxin A. The SLTI and II toxins of E. coli and encoded by lysogenic phage. Its expression is controlled by iron concentration in the growth medium by way of the fur gene and iron box repressor protein binding site. Clostridia difficile toxins A and B also bind to anomeric galactose epitopes on cell membranes and induce membrane associated enzymes and inhibit G protein activation which results in cell death. Tumor cells transfected with a **galactosyltransferase** genes to produce the a .alpha.-Gal epitope are susceptible to lysis by both the Shiga-like toxins and C. difficile toxin. The expression of the .alpha.-Gal epitope is enabled by the transfection of nucleic acids encoding -Gal transferase into tumor cells.

Detail Description Paragraph - DETX:

[0282] Phage display is preferably done using the filamentous phage f88-4 and comprises forming a fusion that results in the C terminus of the "selected" (i.e., inserted gene's) product and the N terminus of the phage protein gVIIp. Peptides of various enterotoxins are expressed in the phage display--most preferably peptides that bind to the SAg receptor on colon carcinoma cells. These peptides retain their capacity to bind to the TCR and to activate T cells. Also contemplated within this invention is phage display of SAg plus nucleic acid encoding synthesis of GalCer and/or the Gal epitope. DNA for synthesis of GalCer is preferably isolated from Sphingomonas paucimobilis; DNA encoding the **galactosyl transferase** for synthesis of Gal is preferably isolated from Klebsiella aerobacter, Serratia, E. coli and Salmonella organisms which naturally produce and express these epitopes. The phage displays are administered in vivo and are capable of initiating a potent immune response to the tumor using the protocols described in Examples 5 and 13 and Section 19, above. These preparations are also useful for activating T cells or NKT cells ex vivo to produce a tumor specific effector cells for use in adoptive immunotherapy (Examples 2-5, 14-16, 18-23).

Detail Description Paragraph - DETX:

[0283] Viral infection of a host cell having the **galactosyl transferase** results in the shedding of virions that express the .alpha.Gal epitope. When a host mammalian cell has been transfected with nucleic acid encoding SAg, the virus can coexpress the Gal epitope and the SAg on its surface. Such a viral construct is administered in vivo to achieve a therapeutic effect, or, in another embodiment, is employed ex vivo to produce tumor specific effector T or NKT cells for use in adoptive immunotherapy of cancer (Examples 2, 3, 7, 15, 16, 18-23).

Detail Description Paragraph - DETX:

[0434] The present invention also includes the additional introduction, into the S/D/t cell of with additional nucleic acids. In one embodiment, the additional nucleic acid encodes the particular **galactosyltransferase** enzyme that catalyze the synthesis of the "heterograft epitope" Gal. In another embodiment, the additional nucleic acid encodes enzymes that synthesize galactosylceramide which is the "natural" epitope recognized by the invariant chain of NKT cells.

Detail Description Paragraph - DETX:

[0467] DNA encoding the **galactosyltransferase** that synthesizes the saccharide structure containing the .alpha.Gal epitope, and gene clusters encoding the biosynthetic pathway for LPS are described in Schnaitman C A, et al., Microbiol. Rev. 57: 655-682 (1993). DNA is extracted from bacteria which biosynthesize these molecules and used to transfect DCs, tumor cells, or S/D/t cells For creation of the GalCer structure, the source of DNA is Sphingomonas paucimobilis organisms. Nucleic acids encoding the pathways for biosynthesis of .beta.-1,3-glucans, peptidoglycans, and protein A have been cloned from insects and Staphylococcus aureus, respectively. These nucleic acids are cloned into suitable expression vectors and introduced into the target cells. Resulting S/D/t cells thus express SAg as well as the anti-tumor motif structure.

Detail Description Paragraph - DETX:

[0655] GLTP is specific for neutral glycosphingolipids and gangliosides but not phospholipids or neutral lipid intermembrane transfers. The protein transfers, by a carrier mechanism, glycolipids with a .beta.-glucosyl or .beta.-galactosyl residue directly linked to either ceramide or diacylglycerol hydrophobic backbone. It has been shown that GLTP facilitates the transfer of glycolipids only when both the donor and acceptor membranes are in the liquid-crystalline state. The GLTP-glycolipid complex is formed as a result of removal of a glycolipid molecule from a membrane and binding to a GLTP molecule. GLTP from pig brain facilitates the transfer of glycosphingolipids which include glucosylceramide, galactosylceramide, lactosylceramide, sulfatide, lactosylceramide-II-sulfate, **globotriaosylceramide,** globotetraosylceramide, globopentaosylceramide, sialosyllactosylceramide, and GM.sub.1 ganglioside. The transfer of Man.beta.1-4Glc.beta.1-ceramide and Man.alpha.1-4Man.beta.1-4Glc.beta.1-ceramide is also facilitated by GLTP from pig brain. In addition to glycosphingolipids, GLTP from pig brain also facilitates the transfer of 3-[Gal.beta.1]-sn-1,2-diacylglycer- ol, 3-[Gal.alpha.1-6Gal.beta.1]-sn-1,2-diacylglycerol, and 3-[Glc.beta.1]-rac-1-2-dipalmitylglycerol. The protein does not facilitate the transfer of 3-[Man.sub.--.alpha.1-3Man.alpha.1]-sn-1,2-dia- cylglycerol, 3-[Glc.alpha.1]-sn-1,2-diacylglycerol, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, cholesterol, or cholesteryl oleate. GLTP from pig brain stimulates the transfer of pyrene-labeled sphingomyelin to a very small but significant extent. GLTP recognizes and

binds the sugar residue directly linked to either ceramide or diacyglycerol in addition to the nonpolar portion of glycolipids.

Detail Description Paragraph - DETX:

[0952] Viral DNA, nucleic acid expression cassettes or plasmids or bacteriophages encoding the constructs given in Table II may be used for in vivo immunization in place of naked DNA. Viruses may also acquire the aGal epitope after transfection into tumor cells which contain the a-**galactosyltransferase** enzyme either naturally or via transfection. The virus must possess the intact N-acetyllactosamine substrate for the **galactosyl-transferase** in order to express the aGal. The viruses shedding from these cells will express the aGal epitope. The virus also contains peptide sequences for SAg and tumor associated antigen acquired from the tumor cells which were previously transfected with nucleic acids encoding SAg and tumor antigen. The shed virus may also express staphylococcal or streptococcal hyaluronidase and capsular polysaccharide sequences obtained from host tumor cell or accessory cells previously transfected with nucleic acids encoding these genes. The shed virus expressing Gal, SAg, hyaluronidase and capsular polysaccharide is capable of initiating a potent tumoricidal response when administered to hosts with established tumors or when used as a tumor vaccine against potential tumors.

Detail Description Paragraph - DETX:

[1019] To prepare glycolipid, phytosphingosine, apolipoprotein, oxyLDL or receptor containing liposomes, 400 mg of galabiosylceramide (Gb2) globotriosylceramide (**Gb3**), globotetraosylceramide (Gb4), galactosylceramide (GalCer), glucosylceramide (GlcCer), phytoshpingosine, oxyLDL or apolipoprotein are dried with 200 mg of phosphatidylethanolamin- e (PE) and 200 mg of phosphatidylserine (PS) under a stream of nitrogen gas. 400 ml of sterile isotonic PBS, pH 7.4, is added to the lipid, and the mixture is sonicated using a water bath sonicator for 30 minutes. Liposome preparations are used immediately.

Detail Description Paragraph - DETX:

[1599] Globotrioslceramides (**GB3**) and globotetraosylceramide (Gb4) are purified from human renal tissue. Briefly, the chloroform/methanol tissue extract is first applied on a Bio-Sil A (Bio-Rad) silica column in chloroform. The column is extensively washed with chloroform, and neutral glycolipids are eluted with acetone/methanol, 9:1 (vol/vol). The neutral glycolipid fraction is then applied on a second Bio-Sil A column in chloroform/methanol, 98:2 (vol/vol). Glycolipids are then resolved with a linear solvent gradient comprising equal weights of chloroform/methanol 15:1 (vol/vol), to chloroform/methanol, 4:1 (vol/vol). Galabiosylceramide (Gb2) or Gal(.alpha.1-4)Gal ceramide from marine sponge may be obtained, for example, from Dr T. Matsubara (Department of Chemistry, Kinki University, Kowakae. Japan).

Detail Description Table CWU - DETL:

13TABLE V Ex vivo Modes of Antigen Presentation to T Cells or NKT Cells to Produce Tumor Specific Effector Cells A. Tumor Cells, Accessory Cells, Accessory Cell/Tumor Cell Hybrids, e.g., DC/Tumor Cell) Transfected with: 1. SAg-encoding nucleic acid 2. SAg-encoding nucleic acid and tumor associated antigen nucleic acids (to include arrays of tumor associated epitopes) 3. SAg nucleic acid and MHC class I or II nucleic acids. 4. SAg-encoding nucleic acid and co-stimulatory nucleic acids. 5. SAg-encoding nucleic acid and adhesion molecule nucleic acids. 6. SAg-encoding nucleic acid and **a-galactosyltransferase** synthetic nucleic acids or xenogeneic species specific nucleic acids. 7. SAg-encoding nucleic acid and chemoattractant nucleic acids 8. SAg-encoding nucleic acid and glycosylceramide synthesis nucleic acids 9. SAg nucleic acid and lipopolysaccharide synthesis nucleic acids 10. SAg-encoding nucleic acid and microbial lipoprotein or polysaccharide or peptidoglycan membrane or capsular synthesis nucleic acids 11. SAg-encoding nucleic acid and SAg receptor nucleic acids 12. SAg-encoding nucleic acid and CD1 receptor synthesis nucleic acids 13. SAg-encoding nucleic acid and CD14 receptor synthesis nucleic acids 14. SAg-encoding nucleic acid and SAg promoter and/or global regulator nucleic acids 15. SAg-encoding nucleic acid and oncogene and/or transcription factor nucleic acids 16. SAg-encoding nucleic acid and angiogenesis factor or receptor nucleic acids 17. SAg-encoding nucleic acid and growth factor receptor nucleic acids18. SAg-encoding nucleic acid and cell cycle protein nucleic acids 19. SAg-encoding nucleic acid and heat shock protein nucleic acids 20. SAg-encoding nucleic acid and chemokine nucleic acids 21. SAg-encoding nucleic acid and cytokine nucleic acids 22. SAg-encoding nucleic acid and tumor suppressor nucleic acids 23. SAg-encoding nucleic acid and antigen processing and trafficking nucleic acids· B. Additional in vitro Stimulatory Agents (preferred receptor) 1. Tumor peptides (Class I or Class II) 2. Tumor peptide-SAg conjugates or fusion proteins (Class I or Class II). 3. Lipopolysaccharide-SAg conjugate (Class II or CD14) a. arabinose b. mycolic acid c. teichoic acid d. muramic acid (Staphylococcal cell wall glycoprotein) e. mannan proteoglycans f. chondroitin-sulfate 4. Glycosylated SAgs. (Class II or mannose) 5. SAg-glycosylceramide conjugates (class II or CD1) a. GalCer conjugate b. .alpha.Gal conjugate 6. SAg-proteosome conjugates 7. SAg or glycosylated SAg or SAg-glycosylcerantide conjugates or SAg- lipopolvsaccharide or SAg-peptidoglycan conjugates coupled to proteosomes 8. SAg or glycosylated SAg or SAg-glycosylceramide conjugates or SAg- lipopolysaccharide conjugates or SAg-peptidoglycan conjugates expressed on or coupled to liposomes 9. Conjugates having having a Superantigen component (polypeptide or nucleic acid) and a partner that is either a single component or a conjugate of 2 or more components (protein, carbohydrate, lipid DNA) as indicated below. Superantigen (Protein or DNA) Partner (Single Component or Conjugate) 1. DNA coding sequence 2. Polypeptide 3. Nucleic acid 4. Tumor associated Peptide 5. Tumor Antigen-MHC protein 6. LPS 7. Lipoarabinomannan 8. Ganglioside 9. Glycosphingolipid 10. Ganglioside-CD1 receptor 11. Glycosphingolipid-CD1 receptor 12. Glycosylceramide (e.g., Gal-Cer) 13. GalCer-CDl receptor 14. Gal 15. Arg-Gly-Asp or Asn-Gly-Arg 16. iNOS 17. Gb2 or **Gb3** or Gb4 18. (Gb2 or **Gb3** or Gb4)-CD1 receptor 19. -GPI-(Gb2 or **Gb3** or Gb4) 20. -GPI-(Gb2 or **Gb3** or Gb4)-CD1 receptor 21. Verotoxin 22. Verotoxin A or B Subunit 23. TFNa receptor peptide homologous to VT 24. CD19 peptide homologous to VT 25. LDL,

VLDL, HDL, IDL 26. Apolipoproteins (e.g., Lp(a), apoB-100, apoB-48, apoE) 27. OxyLDL, oxyLDL mimics, (e.g., 7b-hydroperoxycholesterol, 7b-hydroxycholesterol, 7-ketocholesterol, 5a-6a-epoxycholesterol, 7b-hydroperoxy-choles-5-en-3b-ol, 4-hydroxynonenal (4-HNE), 9-HODE, 13-HODE and chlolesterol-9-HODE) 28. OxyLDL by products (e.g. lysolecithin, lysophosphatidyicholine, malondialdehyde, 4-hydroxynonenal) 29. LDL & oxyLDL receptors (e.g., LDL oxyLDL, acetyl-LDL, VLDL, LRP, CD36, SREC, LOX-1, macrophage scavenger receptors) 30. phytosphingosine, -GPI-phytosphinosine 31. tumor associated lipid antigens 32. glycolipid, proteolipid, glycosphingolipid, sphingolipid with inositoiphosphate-containing head groups, phytoglycolipids, mycoglycolipids, -GPI-sphinosines or GPI-lipids 33. sphingolipids with inositoiphosphate-containing head groups having the general structure: ceramide-P-myoinositol-X with X referring to polar substituents comprising ceranude-p-inositol-mannose, inositol-1-P-(6)mannose(al,2inositol-1P-(1)ceranude, (inositol-P)2-ceramide, inositol-P-inositol-P-ceranude, inositol-P-inositol-P-ceramide. 34. tumor associated glycan antigens consisting of peptidoglycans or glycan phosphotidyinositol (GPI) structures C. STCT or SAg-tumor peptide conjugates are incubated with in vivo immunized T cells or NKT cells for 24 days and then with IL-2 for 2-5 days. D. The tumor specific effector cells are then harvested and injected in doses of 10.sup.10-10.sup.12 every 3-7 days for 1-6 treatments. E. Viruses are transfected into tumor cells, accessory cells, antigen presenting cells, allogeneic or xenogeneic cells. They are pre-programmed with DNA for SAgs alone or in combination with genes given in D. They may also utilize the host genome to produce a new gene product as for example the host **-galactosyltransferase.** Viruses may include the following: 1. Adenoviruses. 2. Vaccinia virus. 3. Equine encephalitis virus. 4. Influenza virus. F. In an additional method, tumor associated antigens are bound to MHC class I positive cells and used to activate T cells. SAg-lipopolysaccharide complexes and SAg-glycosylceramide complexes are bound to CD1 or class II receptors on APCs. In addition, SAg-lipopolysaccharide complexes or SAg-glycosylceramide complexes are presented bound to class II positive APCs. Alternatively, unbound tumor associated antigen/SAg conjugates or fusion products are added at a 0.1 to 200 mg/ml dose for 2 days. This is followed by STCT incubation or by native or mutant SAg treatment for 2 days.

Detail Description Table CWU - DETL:

17 Superantigen (Protein or DNA) Partner (Single Component or Conjugate) 4. DNA coding sequence 5. Polypeptide 6. Nucleic acid 7. Tumor associated Peptide 8. Tumor Antigen-MHC protein 9. LPS 10. Lipoarabinomannan 11. Ganglioside 12. Glycosphingolipid 13. Ganglioside-CD1 receptor 14. Glycosphingolipid-CD1 receptor 15. Glycosylceramide (e.g., Gal-Cer) 16. GalCer-CD1 receptor 17. Gal 18. Arg-Gly-Asp or Asn-Gly-Arg 19 iNOS 20. Gb2 or **Gb3** or Gb4 21. (Gb2 or **Gb3** or Gb4)-CD1 receptor 22. -GPI-(Gb2 or **Gb3** or Gb4) 23. -GPI-(Gb2 or **Gb3** or Gb4)-CD1 receptor 24. Verotoxin 25. Verotoxin A or B Subunit 26. IFN.alpha. receptor peptide homologous to VT 27. CD19 peptide homologous to VT 28. LDL, VLDL, HDL, IDL 29. Apolipoproteins (e.g., Lp(a), apoB-100, apoB-48, apoE) 30. OxyLDL, oxyLDL mimics, (e.g.,. 7b-hydroperoxycholesterol, 7.beta.- hydroxycholesterol, 7-ketocholesterol, 5.alpha.-6.alpha.-epoxycholesterol- , 7.beta.- hydroperoxy-choles-5-en-3b-ol, 4-hydroxynonenal (4-HNE), 9- HODE, 13-HODE and cholesterol-9-HODE) 31.

OxyLDL by products (e.g. lysolecithin, lysophosphatidylcholine, malondialdehyde, 4-hydroxynonenal) 32. LDL & oxyLDL receptors (e.g., LDL oxyLDL, acetyl-LDL, VLDL, LRP, CD36, SREC, LOX-1, macrophage scavenger receptors) 33. phytosphingosine, -GPI-phytosphingosine 34. tumor associated lipid antigens 35. glycolipid, proteolipid, glycosphingolipid, sphingolipid with inositolphosphate -containing head groups, phytoglycolipids, mycoglycolipids, -GPI-sphingosines, -GPI-lipids 36. sphingolipids with inositolphosphate-containing head groups having the general structure: ceramide-P-myoinositol-X with X referring to polar substituents comprising ceramide-p-inositol-mannose, inositol-1-P-(6)mannose(a1,2 inositol-1P-(1)ceramide, (inositol-P)2-ceramide, inositol-P-inositol-P-ceramide, inositol-P-inositol-P-ceramide. 37. tumor associated glycan antigens consisting of peptidoglycans or glycan phosphotidyinositol (GPI) structures

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TITLE: Systems and methods for automated analysis of cells and tissues

PUBLICATION-DATE: November 28, 2002

INVENTOR-INFORMATION:

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APPL-NO: 10/ 062308

DATE FILED: February 1, 2002

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non-provisional-of-provisional 60285155 20010420 US

US-CL-CURRENT: 435/6,382/128 ,435/7.2 ,702/19 ,702/20

ABSTRACT:

Systems and methods for rapidly analyzing cell containing samples, for example to identify morphology or to localize and quantitate biomarkers are disclosed.

[0001] Under 35 USC .sctn.119(e)(1), this application claims the benefit of prior U.S. provisional application _____, filed Apr. 19, 2001, and U.S. provisional application 60/334,723, filed Oct. 31, 2001, the contents of which are incorporated herein by reference. Work described herein was supported in part by funding from the National Institute of Health. The U.S. Government may therefore have certain rights in the invention.

---------- KWIC ---------

Detail Description Paragraph - DETX:

[0078] Biological markers, which may be detected in accordance with the present invention include, but are not limited to any nucleic acids, proteins, peptides, lipids, carbohydrates or other components of a cell. Certain markers are characteristic of particular cells, while other markers have been identified as being associated with a particular disease or condition. Examples of known prognostic markers include enzymatic markers such as

galactosyl transferase II, neuron specific enolase, proton ATPase-2, and acid phosphatase. Hormone or hormone receptor markers include human chorionic gonadotropin (HCG), adrenocorticotropic hormone, carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), estrogen receptor, progesterone receptor, androgen receptor, gC1q-R/p33 complement receptor, IL-2 receptor, p75 neurotrophin receptor, PTH receptor, thyroid hormone receptor, and insulin receptor.

Detail Description Paragraph - DETX:

[0083] Cluster differentiation markers include CD1a, CD1b, CD1 c, CD1d, CD1e, CD2, CD3delta, CD3epsilon, CD3gamma, CD4, CD5, CD6, CD7, CD8alpha, CD8beta, CD9, CD10, CD11a, CD11b, CD11c, CDw12, CD13, CD14, CD15, CD15s, CD16a, CD16b, CDw17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CD32, CD33, CD34, CD35, CD36, CD37, CD38, CD39, CD40, CD41, CD42a, CD42b, CD42c, CD42d, CD43, CD44, CD44R, CD45, CD46, CD47, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD50, CD51, CD52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CDw60, CD61, CD62E, CD62L, CD62P, CD63, CD64, CD65, CD65s, CD66a, CD66b, CD66c, CD66d, CD66e, CD66f, CD68, CD69, CD70, CD71, CD72, CD73, CD74, CDw75, CDw76, **CD77,** CD79a, CD79b, CD80, CD81, CD82, CD83, CD84, CD85, CD86, CD87, CD88, CD89, CD90, CD91, CDw92, CDw93, CD94, CD95, CD96, CD97, CD98, CD99, CD100, CD101, CD102, CD103, CD104, CD105, CD106, CD107a, CD107b, CDw108, CD109, CD114, CD115, CD116, CD117, CDw119, CD120a, CD120b, CD121a, CDw121b, CD122, CD123, CD124, CDw125, CD126, CD127, CDw128a, CDw128b, CD130, CDw131, CD132, CD134, CD135, CDw136, CDw137, CD138, CD139, CD140a, CD140b, CD141, CD142, CD143, CD144, CDw145, CD146, CD147, CD148, CDw149, CDw150, CD151, CD152, CD153, CD154, CD155, CD156, CD157, CD158a, CD158b, CD161, CD162, CD163, CD164, CD165, CD166, and TCR-zeta.

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DOCUMENT-IDENTIFIER: US 20020172944 A1

TITLE: PROTEIN ACTIVITY SCREENING OF CLONES HAVING DNA FROM UNCULTIVATED
MICROORGANISMS

PUBLICATION-DATE: November 21, 2002

INVENTOR-INFORMATION:

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DATE FILED: September 28, 1999

CONTINUED PROSECUTION APPLICATION: This is a publication of a continued prosecution application (CPA) filed under 37 CFR 1.53(d).

RELATED-US-APPL-DATA:

child 09407525 A1 19990928 parent continuation-of 08657409 19960603 US GRANTED parent-patent 5958672 US child 08657409 19960603 US parent continuation-in-part-of 08568994 19951207 US ABANDONED child 08568994 19951207 US parent continuation-in-part-of 08503606 19950718 US GRANTED parent-patent 6004788 US

US-CL-CURRENT: 435/6

ABSTRACT:

Disclosed is a process of screening clones having DNA from an uncultivated microorganism for a specified protein, e.g. enzyme, activity by screening for a specified protein, e.g. enzyme, activity in a library of clones prepared by (i) recovering DNA from a DNA population derived from at least one uncultivated microorganism; and (ii) transforming a host with recovered DNA to produce a library of clones which is screened for the specified protein, e.g. enzyme, activity.

[0001] This application is a continuation-in-part of U.S. application Ser. No. 08/568,994 which was filed on Dec. 7, 1995 (copending) which is a continuation-in-part of U.S. application Ser. No. 08/503,606 which was filed on Jul. 18, 1995 (copending).

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0091] d. Glycoside synthesis using UDP-**galactosyl transferase**

Detail Description Table CWU - DETL:

13TABLE 4 23 4-methyl umbelliferone wherein R = G2 .beta.-D-galactose .beta.-D-glucose .beta.-D-glucoronide **GB3** .beta.-D-cellotrioside .beta.-B-cellobiopyranoside GC3 .beta.-D-galactose .alpha.-D-galactose GD3 .beta.-D-glucose .alpha.-D-glucose GE3 .beta.-D-glucuronide GI3 .beta.-D-N,N-diacetylchitobiose GJ3 .beta.-D-fucose .alpha.-L-fucose .beta.-L-fucose GK3 .beta.-D-mannose .alpha.-D-mannose non-Umbelliferyl substrates GA3 amylose [polyglucan .alpha.1,4 linkages], amylopectin [polyglucan branching .alpha.1,6 linkages] GF3 xylan [poly 1,4-D-xylan] GG3 amylopectin, pullulan GH3 sucrose, fructofuranoside

PGPUB-DOCUMENT-NUMBER: 20020150968

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020150968 A1

TITLE: Glycoconjugate and sugar nucleotide synthesis using solid supports

PUBLICATION-DATE: October 17, 2002

INVENTOR-INFORMATION:

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APPL-NO: 09/ 757846

DATE FILED: January 10, 2001

US-CL-CURRENT: 435/53,435/175 ,435/68.1 ,435/96

ABSTRACT:

This invention relates to methods and compositions for the in vitro production of glycoconjugates. In particular, a preferred production system is provided that comprises a solid support, at least one sugar nucleotide producing enzyme, at least one glycosyltransferase, at least one bioenergetic, and at least one acceptor. The sugar nucleotide producing enzyme(s) is preferably immobilized on the solid support. The glycosyltransferase may be co-immobilized on the solid support with the sugar nucleotide producing enzyme(s), or may be provided to the solid support in solution.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0005] In 1998, Kyowa Hakko Inc. in Japan made a significant breakthrough in large-scale synthesis of carbohydrates (Koizumi, S. et al., Nature Biotech. 1998, 16: 847-850). The key in Kyowa Hakko's technology for the large-scale production of UDP-galactose and Gal.alpha.1,4Lac globotriose was a C. ammoniagenes bacterial strain engineered to efficiently convert inexpensive orotic acid to UTP. When combined with an E. coli strain engineered to over-express UDP-galactose biosynthetic genes including galK (galactokinase), galT (galactose-1-phosphate uridyltransferase), galU (glucose-1-phosphate uridyltransferase), and ppa (pyrophosphatase), UDP-galactose accumulated in the reaction solution. Combining these two strains with another recombinant E. coli strain over-expressing .alpha.1,4-**galactosyltransferase** gene of Neisseria gonorrhoeae, high concentration of globotriose was obtained.

Brief Description of Drawings Paragraph - DRTX:

[0015] FIG. 1 Metabolic biopathway for the synthesis of .alpha.-Gal. Five enzymes are involved including .alpha.1,3GalT (.alpha.1,3-galactosyltransferase, EC 2.4.1.151), GalK (galactokinase, EC 2.7.1.6), GalT (galactose-1-phosphate uridylyltransferase, EC 2.7.7.10), GalU (glucose-1-phosphate uridylyltransferase, EC 2.7.7.9), and PykF (pyruvate kinase, EC 2.7.1.40). Metal cofactors required by individual enzymes are shown.

Detail Description Paragraph - DETX:

[0091] A large number of glycosyltransferases that transfer galactose (**galactosyltransferase**) are known. Breton et al. provides an extensive list of prokaryotic and eukaryotic **galactosyltransferases** and is incorporated herein by reference (J. Biochem. 1998, 123:1000-1009). Another list can be found at http://stanxterm.aecom.yu.edu/glyc-T/galt.ht- m (visited Nov. 21, 2000). Galalactosyltransferases include .alpha.1,2 **galactosyltransferases**, such as Gma12p from yeast (Genbank Acc. No. Z30917), .alpha.1,3 **galactosyltransferases**, such as GGTA1 from mouse (Genbank Acc. No. M26925), .beta.1,4 **galactosyltransferases**, such as GalT-I from human (Genbank Acc. No. X55415), and ceramide **galactosyltransferases**, such as CGT from Man (Genbank Acc. No. U30930). **Galactosyltransferases** that transfer galactose from UDP-Gal to an acceptor molecule include .alpha.1,3GalT, .beta.1,4GalT (LgtB), and .alpha.1,4GalT (LgtC).

Detail Description Paragraph - DETX:

[0128] The inventors also recognize that organisms that naturally express one or more enzymes, or have been engineered to express one or more enzymes, required for a particular glycoconjugate synthesis scheme may be useful. Examples include Escherichia coli which expresses the ceramide glucosyltransferase gene derived from human melanoma cell line SK-Mel-28 (Proc. Natl. Acad. Sci. USA, 1996, 93:4638), human melanoma cell line WM266-4 which produces beta 1,3-**galactosyltransferase** (ATCC CRL 1676), recombinant cell line such as namalwa cell line KJM-1 or the like which contains the beta 1,3-**galactosyltransferase** gene derived from the human melanoma cell line WM266-4 (Japanese Published Unexamined Patent Application No. 181759/94), Escherichia coli (EMBO J., 1990, 9, 3171) or Saccharomyces cerevisiae (Biochem, Biophys. Res. Commun., 1994, 201, 160) which expresses the beta 1,4-**galactosyltransferase** gene derived from human HeLa cells, COS-7 cell line (ATCC CRL 1651) which expresses the rat beta 1,6-N-acetylglucosaminyltransferase gene (J. Biol. Chem., 1993, 268: 15381), Sf9 cell line which expresses human N-acetylglucosaminyltransfera- se gene (J. Biochem., 1995, 118: 568), Escherichia coli which expresses human glucuronosyltransferase (Biochem. Biophys. Res. Commun., 1993, 196: 473), namalwa cell line which expresses human alpha 1,3-fucosyltransferase (J. Biol. Chem., 1994, 269: 14730), COS-1 cell line which expresses human alpha

1,3/1,4-fucosyltransferase (Genes Dev., 1990, 4: 1288), COS-1 cell line which expresses human alpha 1,2-fucosyltransferase (Proc. Natl. Acad. Sci. USA., 1990, 87: 6674), COS-7 cell line which expresses chicken alpha 2,6-sialyltransferase (Eur. J. Biochem., 1994, 219: 375), COS cell line which expresses human alpha 2,8-sialyltransferase (Proc. Natl. Acad. Sci. USA., 1994, 91: 7952), Escherichia coli which expresses beta 1,3-N-acetylglucosaminyltransferase- , beta 1,4-galactosyltransferase, beta 1,3-N-acetylgalactosaminyltransfera- se or alpha 1,4-galactosyltransferase derived from Neisseria (WO 96/10086), Escherichia coli which expresses Neisseria-derived alpha 2,3-sialyltransferase (J. Biol. Chem., 1996, 271: 28271), Escherichia coli which expresses Heilcobacter pylori-derived alpha 1,3-fucosyltransferase (J. Biol. Chem., 1997, 272: 21349 and 21357), and Escherichia coli which expresses yeast-derived alpha 1,2-mannosyltransferase (J. Org. Chem., 1993, 58: 3985). Such organism when further complemented with additional sugar-nucleotide regenerating enzymes will be useful in the methods of the present invention.

Detail Description Paragraph - DETX:

[0179] The versatility of the UDP-Gal regeneration beads is exemplified by the following examples of syntheses of a variety of glycoconjugates (Table 1, entries 3 and 4). A combination of the beads with bovine .beta.1,4galactosyltransferase (Sigma) in solution readily produced Gal.beta.1,4GlcNAc with 92% yield. Gal.beta.1,4GlcNAc is one of the most common sugar sequences existing in a variety of natural glycoconjugates. A combination of the beads with .alpha.1,4galactosyltransferase immobilized on beads produced Gal.alpha.1,4Gal.beta.1, GlcOBn with 86% yield (Table 1, entry 4). This sugar sequence (called globotriose Gb.sub.3) is a trisaccharide portion of globotriaosylceramide, which is the receptor of E. coli derived verotoxin (VT). VT binding to the Gb.sub.3 is believed to be a crucial step in the development of hemorrhagic colitis, and hemolytic uremic syndrome commonly known as `Hamburger disease`. Synthetic Gb.sub.3 derivatives could be effective inhibitors of this interaction and have important pharmaceutical potential. The .alpha.1,4galactosyltransferase (lgtC gene) used to produce the enzyme was cloned from Neisseria meningitidis and expressed in E. coli BL21 (DE3) (Kowal and Wang, unpublished data).

Detail Description Paragraph - DETX:

[0181] The UDP-Gal regeneration beads can be used in combination with multiple galactosyltransferases. For example, both .alpha.1,3GalT and .beta.1,4GalT can be simultaneously immobilized onto the beads to generate specific Gal.alpha.1,3Gal.beta.1,4Glc sequence-producing beads. Using two equivalents 1-.sup.13C labeled galactose and four equivalents of PEP as starting materials, double 1-.sup.13C labeled trisaccharide was produced (Table 1, entry 7) from GlcNAc when enough reaction time (10 days) was given. Disaccharide (1-.sup.13C)Gal.beta.1,4GlcNAc was formed as an intermediate as indicated by TLC during the reaction process. Similarly, using two equivalents of galactose as starting sugar and GlcNAc.beta.1,3Gal.beta.1,3GlcN.sub.3 as an acceptor, pentasaccharide (Table 1, entry 8) was produced with 76% overall yield.

Claims Text - CLTX:

1. An in vitro glycoconjugate-producing system comprising: a solid support; one or more sugar nucleotide producing enzyme(s) selected from the group consisting of GalK, GalT, GalU, PykF, Ndk, PpK, AcK, PoxB, Ppa, PgM, NagE, AgmI, glmU, a GalNAc kinase, a pyrophosphorylase, Ugd, NanA, Cmk, NeuA, Alg2, Alg1, SusA, GalE, GMP, GMD, and GFS; and one or more glycosyltransferase enzyme(s) selected from the group consisting of **galactosyltransferases,** glucosyltransferases, N-acetylglucosaminyltransfe- rases, N-acetylgalactosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, and fucosyltransferases; wherein at least one of said enzymes is immobilized on said solid support.

Claims Text - CLTX:

33. The in vitro glycoconjugate-producing system of claim 1, wherein the glycosyltranferase is .alpha.1,3**-galactosyltransferase**.

PGPUB-DOCUMENT-NUMBER: 20020132320

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020132320 A1

TITLE: Glycoconjugate synthesis using a pathway-engineered organism

PUBLICATION-DATE: September 19, 2002

INVENTOR-INFORMATION:

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APPL-NO: 09/ 758525

DATE FILED: January 10, 2001

US-CL-CURRENT: 435/193,435/101 ,435/200 ,435/320.1 ,435/325

ABSTRACT:

This invention relates to methods and compositions for the production of glycoconjugates. In particular, organisms are provided with at least one heterologous gene encoding an enzyme for regenerating a sugar nucleotide along with at least one glycosyltransterase. Such organisms are useful for the large-scale synthesis of glycoconjugates.

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Summary of Invention Paragraph - BSTX:

[0005] In 1998, Kyowa Hakko Inc. in Japan made a significant breakthrough in large-scale synthesis of carbohydrates (Koizumi, S. et al., Nature Biotech. 1998, 16, 847-850). The key in Kyowa Hakko's technology for the large-scale production of UDP-galactose and Gal.alpha.1,4Lac globotriose was a C. ammoniagenes bacterial strain engineered to efficiently convert inexpensive orotic acid to UTP. When combined with an E. coli strain engineered to over-express UDP-galactose biosynthetic genes including galK (galactokinase), galT (galactose-1-phosphate uridyltransferase), galU (glucose-1-phosphate uridyltransferase), and ppa (pyrophosphatase), UDP-galactose accumulated in the reaction solution. By combining these two strains with another recombinant E. coli strain over-expressing .alpha.1,4-**galactosyltransferase** gene of Neisseria gonorrhoeae, a high concentration of globotriose was obtained.

Brief Description of Drawings Paragraph - DRTX:

[0017] FIG. 1 Metabolic biopathway for the synthesis of .alpha.-Gal. Five enzymes are involved including .alpha.1,3GalT (.alpha.1,3-galactosyltransferase, EC 2.4.1.151), GalT (galactose-1-phosphate uridylyltransferase, EC 2.7.7.10), GalU (glucose-1-phosphate uridylyltransferase, EC 2.7.7.9), and PykF (pyruvate kinase, EC 2.7.1.40). Metal cofactors required by individual enzymes are shown.

Detail Description Paragraph - DETX:

[0105] A large number of glycosyltransferases that transfer galactose (galactosyltransferase) are known. Breton et al. provides an extensive list of prokaryotic and eukaryotic galactosyltransferases and is incorporated herein by reference (J. Biochem. 1998, 123, 1000-1009). Another list can be found at http://stanxterm.aecom.yu.edu/glyc-T/galt.ht- m (visited Jan. 9, 2001). Galalactosyltransferases include .alpha.1,2 galactosyltransferases, such as Gmal2p from yeast (Genbank Acc. No. Z30917), .alpha.1,3 galactosyltransferases, such as GGTA1 from mouse (Genbank Acc. No. M26925), .beta.1,4 galactosyltransferases, such as GalT-I from human (Genbank Acc. No. X55415), and ceramide galactosyltransferases, such as CGT from Man (Genbank Acc. No. U30930). Galactosyltransferases that transfer galactose from UDP-Gal to an acceptor molecule include .alpha.1,3GalT, .beta.1,4GalT (LgtB), and .alpha.1,4GalT (LgtC).

Detail Description Paragraph - DETX:

[0135] In other embodiments, the coding regions of two or more enzymes are linked to create a fusion protein. In preferred embodiments, an epimerase-glycosyltransferase fusion protein is encoded (Chen et al., J. Biol Chem 2000, 275(41):31594-31600). In a more preferred embodiment, the epimerase-glycosyltransferase fusion protein comprises UDP-galactose 4-epimerase and a 1,3-galactosyltransferase.

Detail Description Paragraph - DETX:

[0143] The inventors also recognize that organisms that naturally express one or more enzymes, or have been engineered to express one or more enzymes, required for a particular glycoconjugate synthesis scheme may be useful. Examples include Escherichia coli which expresses the ceramide glucosyltransferase gene derived from human melanoma cell line SK-Mel-28 (Proc. Natl. Acad. Sci. USA., 1996, 93, 4638), human melanoma cell line WM266-4 which produces .beta.1,3-galactosyltransferase (ATCC CRL 1676), recombinant cell line such as namalwa cell line KJM-1 or the like which contains the .beta. 1,3-galactosyltransferase gene derived from the human melanoma cell line WM266-4 (Japanese Published Unexamined Patent Application No. 181759/94), Escherichia coli (EMBO J., 1990, 9, 3171) or Saccharomyces cerevisiae (Biochem, Biophys. Res. Commun., 1994, 201, 160) which expresses the .beta.

1,4-galactosyltransferase gene derived from human HeLa cells, COS-7 cell line (ATCC CRL 1651) which expresses the rat .beta. 1,6-N-acetylglucosaminyltransferase gene (J. Biol. Chem., 1993, 268, 15381), Sf9 cell line which expresses human N-acetylglucosaminyltran- sferase gene (J. Biochem., 1995, 118, 568), Escherichia coli which expresses human glucuronosyltransferase (Biochem. Biophys. Res. Commun., 1993, 196, 473), namalwa cell line which expresses human .alpha. 1,3-fucosyltransferase (J. Biol. Chem., 1994, 269, 14730), COS-1 cell line which expresses human .alpha. 1,3/1,4-fucosyltransferase (Genes Dev., 1990, 4, 1288), COS-1 cell line which expresses human .alpha. 1,2-fucosyltransferase (Proc. Natl. Acad. Sci. USA., 1990, 87, 6674), COS-7 cell line which expresses chicken .alpha. 2,6-sialyltransferase (Eur. J. Biochem., 1994, 219, 375), COS cell line which expresses human .alpha. 2,8-sialyltransferase (Proc. Natl. Acad. Sci. USA., 1994, 91, 7952), Escherichia coli which expresses .beta. 1,3-N-acetylglucosaminyltr- ansferase, .beta. 1,4-galactosyltransferase, .beta. 1,3-N-acetylgalactosaminyltransferase or .alpha. 1,4-galactosyltransferas- e derived from Neisseria (WO 96/10086), Escherichia coli which expresses Neisseria-derived .alpha. 2,3-sialyltransferase (J. Biol. Chem., 1996, 271, 28271), Escherichia coli which expresses Heilcobacter pylori-derived .alpha. 1,3-fucosyltransferase (J. Biol. Chem., 1997, 272, 21349 and 21357), and Escherichia coli which expresses yeast-derived .alpha. 1,2-mannosyltransferase (J. Org. Chem., 1993, 58, 3985). Such organism when further complemented with additional sugar-nucleotide regenerating enzymes will be useful in the methods of the present invention.

Detail Description Paragraph - DETX:

[0234] As shown in FIG. 1, the biopathway for the synthesis of .alpha.-Gal oligosaccharides through UDP-Gal (sugar-nucleotide donor of .alpha.1,3GalT) regeneration from UDP (byproduct of the galactosylation reaction) involves five enzymes, including .alpha.1,3-galactosyltransfera- se (.alpha.1,3GalT, EC 2.4.1.151), galactoskinase (GalK, EC 2.7.7.6), galactose-1-phosphate uridylyltransferase (GalT, EC 2.7.1.10), glucose-1-phosphate uridylyltransferase (GalU, EC 2.7.1.9), and pyruvate kinase (PykF, EC 2.7.1.40). .alpha.1,3GalT catalyzes the synthesis of .alpha.-Gal from UDP-Gal and acceptor (lactose or its derivatives). GalK phosphorylates galactose to Gal-1-P with consumption of one molecule of PEP. GalT transfers UDP from UDP-Glc to the galactose in Gal-1-P to produce UDP-Gal. GalU is responsible for the formation of UDP-Glc from UTP and Glc-1-P. The desired galactosylation was catalyzed by an .alpha.1,3GalT to transfer the galactose from the donor UDP-Gal to an acceptor and produce the oligosaccharide with the formation of byproduct UDP. PykF recycles UDP to UTP with the consumption of another molecule of PEP.

Detail Description Paragraph - DETX:

[0235] To obtain active enzymes using recombinant techniques, each of the enzymes involving in the synthetic pathway of .alpha.-Gal was individually cloned and overexpressed with a N-terminal His.sub.6-tag introduced by pET15b vector system. The expression of the enzyme was controlled by a T7 lac promoter and induced by 400 .mu.M of IPTG

(isopropyl-1-thio-.beta.-D-galactopyranoside). Purification of the enzymes was achieved by passing through a Ni.sup.2+-NTA affinity column. An SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) of individual clones indicated that the expression level of each of the four proteins including GalK (A), GalT (B), GalU (C) and PykF (D) was high in pET15b system. The target protein presented more than 80% of the total soluble protein in the host strain. The expression of recombinant .alpha.1,3-**galactosyltransferase** was described in Fang et al. (1998).

Detail Description Paragraph - DETX:

Production of Globotriose (**Gb3**) by Recombinant E. coli Using PykF

Detail Description Paragraph - DETX:

[0260] LgtC catalyzes the transfer of galactose from the donor UDP-Gal to an acceptor and produce the oligosaccharide with the formation of byproduct UDP. Repeatly, LgtC was overexpressed in a high yield as an active soluble form in the cell lysate. About 300 U purified enzyme can be obtained from 1 liter E. coli culture. Globotriose (Gb.sub.3, the sugar sequence of Gal.alpha.1,4Gal.beta. 1,4Glc) is a trisaccharide portion of **globotriaosylceramide,** the receptor of E. coli-derived verotoxin (VT). VT binding to the Gb.sub.3 is believed to be a crucial step in the development of hemorrhagic colitis, and hemolytic uremic syndrome commonly known as `Hamburger disease` (Lingwood, Nephron. 1994, 66, 21-28. Lingwood, C. A. Biochim. Biophys. Acta 1999, 1455, 375. Peter, M. G.; Lingwood, C. A. Biochim. Biophys. Acta 2000, 1501, 116. Barnett, F. D.; Abul-Milh, M.; Huesca, M.; Lingwood, C. A. Infect. Immun. 2000, 68, 3108. Lingwood, C. A. Biosci. Rep. 1999, 19, 345.). Gb.sub.3 plays a direct role in Shiga toxin entry into the cell though the interaction of B-subunit of Shiga toxins and Gb.sub.3. (Lindberg et al., J. Biol. Chem. 1987, 262, 1779-1785.) Synthetic Gb.sub.3 derivatives could be effective inhibitors of these interactions and have important pharmaceutical potential. Gb.sub.3 was also identified as P.sup.k blood group antigen (Marcus et al., Semin. Hematol. 1981, 18, 63-71.) and was found in the LOS (lipooligosaccharides) of the pathogens Neisseria meningitidis immunotype L1 and N. gonorhoeae (Scholten et al., Med. Microbiol. 1994, 41, 236-243. Jennings et al., Mol. Microbiol. 1995, 18, 729-740.). Large amount of Gb.sub.3 is essential for the experimental and clinical research on preventing pathogen invasion.

Claims Text - CLTX:

14. The vector of claim 1, wherein the one or more glycosyltransferase(s) is selected from the group consisting of a **galactosyltransferase,** a glucosyltransferase, an N-acetylglucosaminyl transferase, an N-acetylgalactosaminyl transferase, a glucuronyltransferase, a sialyltransferase, a mannosyltransferase, and a fucosyltransferase.

Claims Text - CLTX:

15. The vector of claim 14, wherein the **galactosyltransferase** is selected from the group consisting of LgtB and LgtC.

Claims Text - CLTX:

36. The vector of claim 35, wherein the glycosyltranferase is .alpha.-1,3-**galactosyltransferase**.

PGPUB-DOCUMENT-NUMBER: 20020086279

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020086279 A1

TITLE: Protein activity screening of clones having DNA from uncultivated microorganisms

PUBLICATION-DATE: July 4, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
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APPL-NO: 09/ 875412

DATE FILED: June 6, 2001

RELATED-US-APPL-DATA:

child 09875412 A1 20010606 parent continuation-of 08988224 19971210 US UNKNOWN

US-CL-CURRENT: 435/4

ABSTRACT:

Disclosed is a process of screening clones having DNA from an uncultivated microorganism for a specified protein, e.g. enzyme, activity by screening for a specified protein, e.g. enzyme, activity in a library of clones prepared by (i) recovering DNA from a DNA population derived from at least one uncultivated microorganism; and (ii) transforming a host with recovered DNA to produce a library of clones which is screened for the specified protein, e.g. enzyme, activity.

RELATED APPLICATIONS

[0001] This application is a divisional application of U.S. patent application Ser. No. 08/657,409, which was filed on Jun. 3, 1996, which was a continuation-in-part of U.S. application Ser. No. 08/568,994 which was filed on Dec. 7, 1995 (copending) which is a continuation-in-part of U.S. application Ser. No. 08/503,606 which was filed on Jul. 18, 1995 (copending).

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Detail Description Paragraph - DETX:

[0092] d. Glycoside synthesis using UDP-**galactosyl transferase**

Detail Description Table CWU - DETL:

5TABLE 4 43 4-methyl umbelliferone wherein R = G2 .beta.-D-galactose .beta.-D-glucose .beta.-D-glucuronide **GB3** .beta.-D-cellotrioside .beta.-D-cellobiopyranoside GC3 .beta.-D-galactose .alpha.-D-galactose CD3 .beta.-D-glucose .alpha.-D-glucose GE3 .beta.-D-glucuronide GI3 .beta.-D-N,N-diacetylchitobiose GJ3 .beta.-D-fucose .alpha.-L-fucose .beta.-L-fucose GK3 .beta.-D-mannose .alpha.-D-mannose non-Umbelliferyl substrates GA3 amylose [polyglucan .alpha.1,4 linkages], amylopectin [polyglucan branching .alpha.1,6 linkages] GF3 xylan [poly 1,4-D-xylan] GG3 amylopectin, pullulan GH3 sucrose, fructofuranoside

US-PAT-NO: 6482586

DOCUMENT-IDENTIFIER: US 6482586 B1

TITLE: Hybrid compositions for intracellular targeting

DATE-ISSUED: November 19, 2002

INVENTOR-INFORMATION:

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APPL-NO: 08/ 975953

DATE FILED: November 21, 1997

PARENT-CASE:

CROSS-REFERENCE TO RELATED APPLICATIONS This application claims priority under 35 U.S.C. 119(e) to co-pending U.S. provisional application Serial No. 60/031,668, filed Nov. 22, 1996; No. 60/061,050, filed Oct. 3, 1997; and No. 60/061,044, filed Oct. 4, 1997; the contents of all of which are hereby incorporated by reference.

US-CL-CURRENT: 435/4; 514/2 ; 530/350 ; 536/23.1

ABSTRACT:

Hybrid compounds comprising a first domain and a second domain are provided. The first domain and the second domain are preferably covalently linked, and the first domain comprises a domain which is capable of specific binding to Gb.sub.3 ; and the second domain comprising a moiety selected from the group consisting of drug moiety, a nucleic acid, a probe, a polypeptide, and a hook, with the proviso that the second domain is not a verotoxin or a fragment thereof. Mehtods of preapring and using the hybrid compounds are also provided.

9 Claims, 2 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 1

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Brief Summary Text - BSTX:

The E coli derived family of verotoxins (or Shiga-like toxins) comprise VT1, VT2 and VT2c, which are involved in the etiology of microvascular disease in man (8), primarily in the very young and elderly (9), and VT2e which causes edema disease in pigs (10). The glycolipid **globotriaosylceramide** (galal-4galbl-4glc cer.-Gb.sub.3) at the plasma membrane is the specific receptor for all verotoxins and mediates the internalization of verotoxin (VT1) into susceptible cells by capping and receptormediated endocytosis (RME) (11). Verotoxin is the only glycolipid binding ligand that is internalized into eukaryotic cells by means of RME (12-14). In addition to receptor concentration, both heterogeneous fatty acid composition of Gb.sub.3 (15, 16) and phospholipid chain length within the phospholipid bilayer (17) play important roles in binding and internalization of VT. Molecular modeling studies of the Gb.sub.3 binding site in the B subunit (18) show that different conformers of membrane Gb.sub.3 may bind in different sites. Such conformers may be related to the Gb.sub.3 fatty acid content and membrane phospholipid microenvironment (18-20).

Detailed Description Text - DETX:

In one aspect, the invention provides a hybrid compounds. The hybrid compound includes a first domain and a second domain; the first and second domains are, preferably, covalently linked. The first domain is a binding domain capable of specific binding to **globotriaosylceramide** (Gb.sub.3) and being internalized into a cell which expresses Gb.sub.3 on the cell surface. The second domain is a functional domain which includes a molecular moiety which is to be delivered into the cell, e.g., to the cell nucleus. The second domain is preferably not a verotoxin, a verotoxin subunit, or a fragment thereof. The second domain can be, for example, a drug moiety (e.g., a drug molecule bound to the first domain), a nucleic acid (e.g., a gene which encodes an exogenous protein, or a nucleic acid which regulates gene expression in a cell, such as antisense nucleic acid, repressors, or trans activators), a probe (such as a fluorescent probe), a protein, and the like. The second domain can also be a domain which functions as a handle or hook for complexation or binding to another moiety or moieties. For example, the second domain can be a member of a specific binding pair (such as biotin/streptavidin, hormone/receptor, binding protein/ligand, and the like), which can be complexed with or bound to the other member of the specific binding pair, which can, in turn, be bound to a moiety which is desired to be delivered into the cell.

Detailed Description Text - DETX:

however, in the presence of butyrate, VT-B is transported to elements of the endoplasmic reticulum and/or the nuclear envelope. As is also described in more detail hereinbelow, this effect is believed to be due, at least in part, to alterations in the fatty acid composition of the cell surface glycolipid Gb.sub.3. The invention contemplates the selective transport of a hybrid compound of the invention to a selected location in the cell, e.g., the nuclear membrane or nucleus. This feature of the invention is especially useful for gene therapy applications (or antisense treatments) in which the nuclear genome, rather than the DNA in the cytoplasm or cytoplasmic organelles, is to

be targeted. Accordingly, in certain embodiments, the invention contemplates treatment of a cell with a compound (e.g., butyrate), or under conditions, capable of effecting a change in the fatty acid composition of **Gb3**, and thereby promoting selective transport of a hybrid compound of the invention to a pre-selected intracellular location (e.g., the nucleus).

Detailed Description Text - DETX:

The first domain of the hybrid compounds of the invention comprises a domain which is capable of specific binding to **globotriaosylceramide** (Gb.sub.3), and is capable of being internalized into a cell which expresses Gb.sub.3 on the cell surface; such a domain will for convenience sometimes be referred to herein as a "VT binding domain" although, as described herein, first domains suitable for use in the invention are not limited to verotoxins or fragments thereof. Domains suitable for use as a first domain of a hybrid compound of the invention include native verotoxins (VTs), subunits of verotoxins (e.g., VT-B subunit) which bind to Gb.sub.3, and polypeptides comprising amino acid sequences homologous to and/or derived from the amino acid sequence of a native VT binding domain, which can include more, fewer (e.g., a deletion or truncation), or an equal number (e.g., point mutations) of amino acids than a full length VT binding domain protein, while retaining substantial specific binding affinity for Gb.sub.3 (or Burkitt's lymphoma associated antigen (BLA) (Nudelman, et al. Science 220: 509 (1983), also known as the B-cell differentiation antigen **CD77**). Thus, a "VT binding domain", as used herein, refers to the Gb.sub.3 receptor binding subunit of verotoxins or homologous domains which have Gb.sub.3 binding activity. It will be appreciated that certain proteins or polypeptides are known which have substantial homology to verotoxin binding domains (e.g., CD19, a 95 kDa immunoglobulin superfamily integral membrane glycoprotein present on the cell surface of human B lymphocytes from the early stage of B-cell development to the terminal differentiation of B-cells to plasma cells (Nadler, et al. J. Immunol. 131: 244-250 (1983); Lingwood, C. A. (1996) Trends in Microbiol. 4(4):147-153; Maloney, M. D. and Lingwood, C. A. (1994) J. Exp. Med. 180: 191-201; Nyholm, P. G., Magnusson, G. and Lingwood, C. (1996) Chem. Biol. 3:263-275) and can bind to Gb.sub.3 or **Gb3**-like cell surface moieties; use of such homologous proteins or polypeptides is contemplated in the hybrid compounds of the invention. In one embodiment, the first domain of a hybrid compound of the invention is at least about 30%, 40%, more preferably at least about 50%, 60%, even more preferably at least about 70%, 80%, yet even more preferably at least about 90%, and most preferably at least about 95% (or more) homologous to a Gb.sub.3 binding domain of a native verotoxin (or verotoxin subunit). Typically, biologically active portions comprise a domain or motif with at least one activity of a VT binding domain. A biologically active portion of a VT binding domain protein can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length.

Detailed Description Text - DETX:

The invention provides methods and compositions useful for targeting molecules to a cell. The cell can be any cell which expresses CD19 on the cell surface and in which CD19 can be internalized, e.g., by interacting with **CD77** as

described herein. Exemplary cells include dendritic cells, B cells, and the like. Suitable cells preferably express **CD77** at The cell surface.

Detailed Description Text - DETX:

In another aspect, the invention provides methods for targeting a moiety for internalization into a CD19-containing cell. The method includes the step of contacting the cell with a CD19-binding moiety bound to a moiety which is to be internalized into the cell (a "targeted" moiety), such at the targeted moiety is internalized into the cell. The targeted moiety can be any moiety which is to be delivered into the cell, e.g., a toxin (e.g., for killing the cell), a polynucleotide, e.g., a gene, (e.g., for genetic modification, e.g., for expression of a gene product in the cell), a protein or peptide (e.g., an antibody or antigen), and the like. The CD19-binding moiety can be, e.g., an anti-CD19 antibody, as described supra. The targeted moiety can be bound or conjugated to the CD19-binding moiety as described above. It will be appreciated that more than one targeted moiety can be bound to a single antibody. An antibody/targeted moiety conjugate preferably does not prevent internalization of CD19 when the conjugate binds to CD19 and the complex subsequently binds to **CD77**.

Detailed Description Text - DETX:

In another aspect, the invention provides a method for presenting an antigen to a cell. The method can be used, e.g., for vaccination. The method includes the step of contacting the cell with a CD19-functional moiety bound to an antigen or antigenic moiety (e.g., an antigen/CD19 conjugate), such that the antigen is presented to the cell. The antigen/CD19 conjugate preferably is capable of modulating (e.g., stimulating) a response in the cell, such as a B cell, when the conjugate contacts the cell, e.g., when the conjugate is administered to a subject (such as a mammal, including a human). The CD19 portion of the CD19 conjugate can comprise native CD19, or a portion of CD19 which has CD19 activity, i.e., a portion or fragment of CD19 which retains the ability to bind to **CD77** and to be endocytosed (along with the antigen portion) into the cell. The CD19 portion of the conjugate can also be a polypeptide which has at least 60% homology to all or a portion of native CD19, more preferably at least 70%, 80%, 90%, or 95% homology to all or a portion of native CD19. In certain preferred embodiments, the CD19 portion of the conjugate comprises the N-terminal portion of CD19. In certain embodiments, the CD19 portion of the conjugate comprises the portion of CD19 which includes sequence homology to VT-B, e.g., the glycolipid receptor portion (Lingwood 1996b, Maloney and Lingwood 1994, Nyholm et al. 1996). It will be appreciated that the cell can, but need not, express CD19, but preferably will express **CD77** at the cell surface. The antigen portion can comprise any antigenic moiety as described above, preferably a protein or peptide from a pathogenic organism such as a bacterium, virus, or parasite. The antigen portion preferably is not a polypeptide sequence of native CD19. The antigen/CD19 conjugate can be produced by methods well known to the skill artisan, including the methods described supra. The antigen/CD19 conjugate can be a fusion protein, preferably a fusion protein which includes, at its N-terminus, an N-terminal portion of CD19, and at the C-terminus of The fusion protein, an antigenic

portion.

Detailed Description Text - DETX:

In another aspect, the invention provides methods for targeting a moiety for internalization into a cell. The method includes the step of contacting the cell with a CD19-functional moiety (e.g., as described above) bound to a moiety which is to be internalized into the cell (a "targeted" moiety), such that the targeted moiety is internalized into the cell. The targeted moiety can be any moiety which is to be delivered into the cell, e.g., a toxin (e.g., for killing the cell), a polynucleotide, e.g., a gene, (e.g., for genetic modification, e.g., for expression of a gene product in the cell), a protein or peptide (e.g., an antibody or antigen), and the like. The CD19-functional moiety can be, e.g., a native CD19, or a portion of CD19 which has CD19 activity, or a polypeptide which is substantially homologous (e.g., at least 60%, 70%, 80%, 90%, or 95% homologous) to a portion of native CD19, as described supra. The targeted moiety can be bound or conjugated to the CD19 moiety as described above. It will be appreciated that more than one targeted moiety can be bound to a single CD19 moiety. A CD19 moiety/targeted moiety conjugate preferably can be internalized into the cell when the conjugate binds to **CD77** at the cell surface.

Detailed Description Text - DETX:

The invention also relates to methods for targeting a moiety for internalization into a **Gb3**-containing cell. The method includes the step of contacting the cell with a hybrid compound of the invention, wherein the hybrid compound includes the moiety for internalization, such that the hybrid compound is internalized into the cell. The moiety can be any moiety which is to be delivered into the cell, e.g., a toxin (e.g., for killing the cell), a polynucleotide, e.g., a gene, (e.g., for genetic modification, e.g., for expression of a gene product in the cell), a protein or peptide (e.g., an antibody or antigen), and the like. The Gb.sub.3 -binding moiety can be, e.g., an anti-Gb.sub.3 antibody, as described supra. The targeted moiety can be bound or conjugated to the **Gb3**-binding moiety as described above.

Detailed Description Text - DETX:

After trypsinization, cells (.about.1.times.10.sup.6) were washed with PBS three times, resuspended in a minimum volume, and extracted with 20 volumes of chloroform/methanol (C/M) 2:1 by vol. The extract was partitioned against water and the lower phase partitioned again against theoretical upper phase. The combined lower phase was then evaporated, saponified with 1 N NaOH in methanol and glycolipids reextracted as above . The dried lower phase was dissolved in CM 98:2 and separated by silica chromatography(32). The column was washed extensively with chloroform and glycolipid eluted in acetone/methanol (9:1 by vol.). **Gb3** present was detected by tlc overlay binding with VT binding domain1 (16).

Detailed Description Text - DETX:

The pattern of FITC-VT binding domain1 B localization was completely distinct in the SF-539 and XF-498 astrocytoma cell lines, despite comparable **Gb3** content and cell surface binding at 4.degree. C. In the more VT binding domain sensitive SF-539 cells, at 37.degree. C., intracellular FITC-VT binding domain1-B accumulated around the nucleus and apparently within the nucleus. However, the intracellular localization of FITC-VT binding domain1-B in XF-498 cells was in a juxtanuclear location, consistent with Golgi localization. Double labeling confocal microscopy verified the targeting of VT binding domain 1 B to the the nuclear envelope/ER and nucleus in SF-539 cells. In SF-539 cells, RITC-B also colocalized with anti BIP (GRP 78), a marker for the ER(41) as a ring around the nucleus. The punctate staining for VT binding domain1B and BIP for the most part was coincident, however some BIP staining showed no corresponding toxin localization and vice versa. The latter result is likely due to VT binding domain1B localization in part, in intermediate compartment vesicles (between Golgi and ER)(42). In addition, intranuclear staining is clearly seen for VT binding domain1 B but not for BIP. Staining for ERGIC 53, a marker of the intermediate compartment vesicles in part, colocalized with VT binding domain1B staining. In contrast, no nuclear staining was seen for XF-498. Double labeling confocal microscopy showed that the juxtanuclear structure labeled in XF 498 cells was colocalized with Con A labeled Golgi. VT binding domain1B was restricted to the Golgi and did not localize with the additional Con A staining of the ER around the nucleus.

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Maloney, M.D. et al. "CD19 Has a Potential **CD77** (Globotriaosyl Ceramide)-binding Site with Sequence Similarity to Verotoxin B-subunits: Implication of Molecular Mimicry for B Cell Adhesion and Enterohemorrhagic Escherichia coli Pathogenesis" J. Exp. Med. 180:191-201 (1994).